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Abstract

The studies in this grant are designed to assess the role of TIMP-1 in invasion and metastasis with emphasis on the Ras signaling pathway. We have previously provided evidence that TIMP-1 may induce epithelial to mesenchymal transition in MCF10A cells. In order to further assess the role of TIMP-1 in breast cancer development, we created several MCF-7 cell lines which overexpress TIMP-1. Using low, medium and high overexpressing clones we determined that high levels of TIMP-1 are directly correlated with expression of VEGF. Interestingly, TIMP-1 had no effect on proliferation of MCF-7 cells. In addition, the TIMP-1 clones demonstrated no difference in invasion compared to vector control cells. It was observed by immunofluorescence that TIMP-1 overexpressing clones had a significantly higher number of multi-nucleated cells. Studies are underway to assess the cause of this phenomenon. We are currently assessing downstream signaling affected by TIMP-1 overexpression. We have observed no changes in FAK activation but increases in the MAPK pathway. Using cytokine protein arrays, we have observed expression changes in several cytokines in the TIMP-1 overexpressing clones, including CSF-1, IL-10 and IGFBP-2. We are currently confirming these results. We have also begun *in vivo* studies by injecting the MCF-7 vector control and 3 clones with varying TIMP-1 levels into mouse mammary fat pads. Results are expected in approximately a month. These combined results provide further evidence that TIMP-1 has a role in tumorigenesis other than inhibition of MMPs.

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INTRODUCTION

The goal of our research is determine how TIMP-1 contributes to breast cancer progression. This MMP inhibitor has historically been considered an anti-cancer protein, but a number of published studies now demonstrate this protein can also act to promote cancer progression. We hypothesize that TIMP-1 can stimulate invasion of breast tumor cells via activation of Ras and other downstream pathways. We further propose that a new version of TIMP-1 can be genetically engineered that will still inhibit MMP function but will be inactivate in promoting cancer progression. The following represents a one year progress report of our studies for the second year. Within the next few months, we will have completed Task 1 and Task 2 Items and will focus on Task 3 items.

BODY (Review of data obtained July 1, 2004 to June 30, 2005)

Overview of this Section:

We received reviewers' comments back on our paper entitled "TIMP-1 Induces Epithelial to Mesenchymal Transition in MCF10A Breast Epithelial Cells" that was submitted to Cancer Research. Although they found the study compelling, their main concern was the incomplete nature of the story. Accordingly, we are attempting to address some of their concerns. For instance, we are trying to generate new MCF10A clones that conditionally overexpress TIMP-1, since the clones sent by our collaborator Dr. Kim (Wayne State University) stopped expressing TIMP-1. In addition, we are attempting to reverse the EMT observed in TIMP-1 overexpressers by using siRNA approaches to downregulate levels of TIMP-1 (see below). Upon completion of these new studies, we will resubmit the paper for publication.

The following section summarizes our approaches and successes over the last year. The sections are divided into a description of data that we are confident we can publish and a section of preliminary data that may lead to one or more publications.

I. Publishable Data

Generation of TIMP-1 MCF-7 Overexpressing Cell-Lines (Relevant to Task 1a, 1b, 1c)

One of our original tasks was to receive from Dr. Thorgeirson MCF-7 tumor cells that overexpressed TIMP-1. Unfortunately, he told us the cell-lines were no longer available. Therefore, we needed to generate our own cells overexpressing TIMP-1. We have successfully completed this task all have 3 sets of overexpressing pools as well as clones that overexpress low (< 250 ng/ml), intermediate (250 – 500 ng/ml) and high (> 500 ng/ml) amounts of TIMP-1. As described below we have begun to analyze these cell-lines in terms of signaling, morphology and changes in motility and invasion.

TIMP-1 Overexpressing MCF-7 Cells Oversecrete VEGF (Relevant to Task 2)

Various cell-lines and pools of overexpressing cells were analyzed for the production of VEGF, a protein known to be important in the regulation of angiogenesis. Fresh media was placed on these cells, and harvested 24 hours later. We used a commercial ELISA kit to measure the level of VEGF. Figure 1 indicates that the extracellular level of VEGF correlated significantly with the level of TIMP-1. Our results are consistent with previous published results and support an important role for TIMP-1 overexpression and the regulation of tumor angiogenesis (see below). We do not yet know the intracellular signaing pathways induced by TIMP-1 to control expression of VEGF.

TIMP-1 Overexpressing Cells Show Defects in Cytokinesis but are Apparently Normal in Proliferation (Relevant to Task 2)

Microscopic examination of MCF-7 tumor cells overexpressing TIMP-1 revealed that as compared to control cells, many of the tumor cells appeared flatter and larger. Fluorescent staining of nuclei and F-actin revealed that many of the TIMP-1 expressing cells had multiple nuclei, and defective F-actin distribution as compared to control cells (Figure 2). This was an unexpected finding, since only a few publications have indicated that TIMP-1 may affect activity involving nuclei. It is conceivable that TIMP-1 overexpression could affect chromosome stability also, and thus contribute to carcinogenesis by inducing mutations. Interestingly, standard proliferation assays indicated that the growth characteristics of control and all of the TIMP-1 overexpressing clones appeared the same, consistent with published results (5).

Analysis of Signaling Pathways in MCF-7 Breast Tumor Cells Overexpressing TIMP-1 (Relevant to Task 2)

No data is available concerning the signaling pathways altered by overexpression of TIMP-1 in MCF-7 cell-lines. Therefore, western blot analysis was performed to determine the affect of TIMP-1 expression on pathways known to contribute to cancer progression. We examined low, medium and high overexpressing clones and found that the PI 3-kinase pathway was not altered in activity as determined by the lack of change in the level of phospho-Akt. Also, focal adhesion kinase (FAK) was not activated in cells overexpressing TIMP-1. This is in contrast to what Dr. Kim observed for MCF10A cells (6,7). We also observed that the MAPK and p38 kinase pathways were slightly elevated in activity in clones expressing medium levels of TIMP-1.

Analysis of Invasion of MCF-7 cells Overexpressing TIMP-1 (Relevant to Task 1)

We used the standard filter insert method coupled with Matrigel to determine if cells overexpressing TIMP-1 were more invasive *in vitro*. None of the TIMP-1 overexpressing clones appeared more invasive than the control cells, a result which does not support our hypothesis that TIMP-1 regulates invasion. However, this is only one cell-line and we have not examined the role of TIMP-1 in regulating invasion in anim als.

II. Additional Preliminary Data

Levels of TIMP-1 Protein are Higher in a Significant Percentage of Invasive Ductal Carcinoma Cells in Humans.

A number of studies have been published demonstrating that the levels of TIMP-1 mRNA or protein (based on E LISA assays) increase in advanced breast cancers (1-4). Unfortunately, only a few conflicting studies have been published using immunohistochemical staining. Therefore, we initiated the following study. We purchased tissue arrays from Folio containing 80 total cores with 35 cases of infiltrating ductal carcinoma with patient matched normal tissue. We performed TIMP-1 immunohistochemistry on the arrays. TIMP-1 was rarely expressed in normal tissue. Of the 35 patients, 7 (20%) had higher levels of TIMP-1 in the tumor cores compared to the patient matched normal tissue. We are currently performing IHC on the arrays with antibodies to VEGF, CSF-1, IL-10 and IGFBP-2 to assess potential correlations between TIMP-1 levels and the listed cytokines induced by TIMP-1 *in vitro* (see below).

TIMP-1 Overexpressing MCF-7 Cells, Oversecrete a Subset of Cytokines

Using cytokine protein array s, we have observed expression changes in several cytokines in the TIMP-1 overexpressing clones, including CSF-1, IL-10 and IGFBP-2. We are currently confirming these results. TIMP-1 induced increases in the levels of CSF-1 is very exciting since

this protein plays an important role in activating macrophages and it contributes to tumor invasion and metastasis. IL-10 is an important cytokine regulating T-cell activity while IGFBP-2 has been implicated in breast cancer.

Animal Studies to Determine if TIMP-1 Overexpressing Cell-Lines are more Angiogenic and Invasive *in vivo* (Relevant to Task 1c)
We have also begun *in vivo* studies by injecting the MCF-7 vector control and 3 clones with varying TIMP-1 levels into mouse mammary fat pads. Results are expected in app roximately a month. We predict that the TIMP-1 overexpressing tumor cells will be more vascularized and invasive than the control cells.

Use of siRNA to Downregulate Expression of TIMP-1 in MB-MDA-231 Tumor Cells Overexpressing TIMP-1.
Studies were proposed (Task 1) to examine the consequences on invasion of tumor cells in which TIMP-1 levels were reduced. We have received 2 siRNA oligonucleotides from BD/Clonetech that have been demonstrated to reduce levels of TIMP-1 protein by greater than 80% following transfection. We soon will be transfecting these oligonucleotides into MB-MDA-231 breast cancer cells and measuring changes in the invasion rates *in vitro*. These breast cancer cells express high levels of TIMP-1 and they are very invasive. If TIMP-1 plays a positive role in regulating invasion, we predict that reducing the levels of this protein will result in a decrease in invasion. Subsequent studies will involve downregulating TIMP-1 in stable clones and examining invasion and metastasis *in vivo*.

KEY RESEARCH ACCOMPLISHMENTS:

1. Generated multiple MCF-7 clonal cell-lines overexpr essing TIMP-1.
2. Determined that TIMP-1 overexpression correlates with overexpression of VEGF.
3. Demonstrated that TIMP-1 overexpression may result in defects in cytokinesis leading to multinucleated cells.
4. Demonstrated that the MAPK and p38 signaling pathw ays were induced in MCF-7 breast tumor cells overexpressing TIM P-1, while no changes were observed for FAK or PI-3K.
5. Demonstrated that MCF-7 TIMP-1 overexpressing cells did not proliferate faster or invade better than contr ol cells.
6. Demonstrated that MCF-7 TIMP-1 overexpressing cells secreted higher levels of M-CSF, IL-10 and IGF BP-2 than control cells.
7. Determined using IHC approaches that levels of TIMP-1 protein were elevated in a significant percentage of invasive breast cancers.

REPORTABLE OUTCOMES:

RLH Bigelow, JA Cardelli. 2004. TIMP-1 Induces Epithelial to Mesenchymal Transition in MCF10A Breast E pithelial Cells. Abstract for the *"95th meeting for the American A ssociation for Cancer Research"*. Orlando, FL.

RLH Bigelow, JA Cardelli. 2004. TIMP-1 Induces Epithelial to Mesenchymal Transition in MCF10A Breast E pithelial Cells. Abstract and poster for Era of Hope Breast Cancer Research Meeting.

RLH Bigelow, JA Cardelli. TIMP-1 Induces Epithelial to Mesenchymal Transition in MCF10A Breast Epithelial Cells. Manuscript in preparation for resubmission.

CONCLUSIONS

In conclusion, we have demonstrated that TIMP-1 overexpression in MCF-7 cells has no effect on proliferation or invasion. TIMP-1 levels were found to be directly correlated with VEGF levels and cytokine arrays demonstrated higher levels of CSF-1, IL-10 and IGFBP-2 in the TIMP-1 overexpressing clones. Interestingly, the TIMP-1 clones had a higher percentage of multi-nucleated cells. Additionally, immunohistochemistry of tissue arrays revealed that TIMP-1 was overexpressed in a significant percentage of infiltrating ductal carcinomas compared to patient matched controls. We are currently doing *in vivo* mouse studies with MCF-7 xenografts to assess the role of TIMP-1 in tumor growth, vascularization, invasion and metastasis. These results further suggest a role for TIMP-1 in the development of the malignant phenotype

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FIGURES

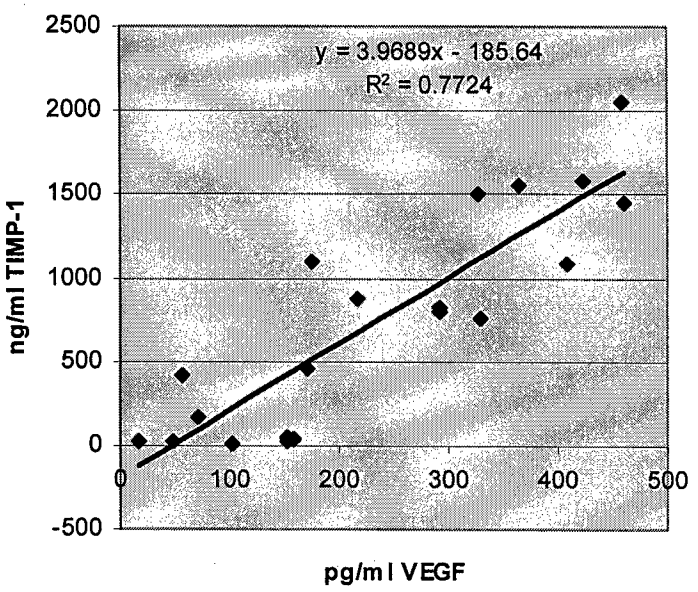

Figure 1 Various TIMP-1 expressing clones and pools of MCF-7 tumor cells were analyzed for the expression of TIMP-1 and VEGF.

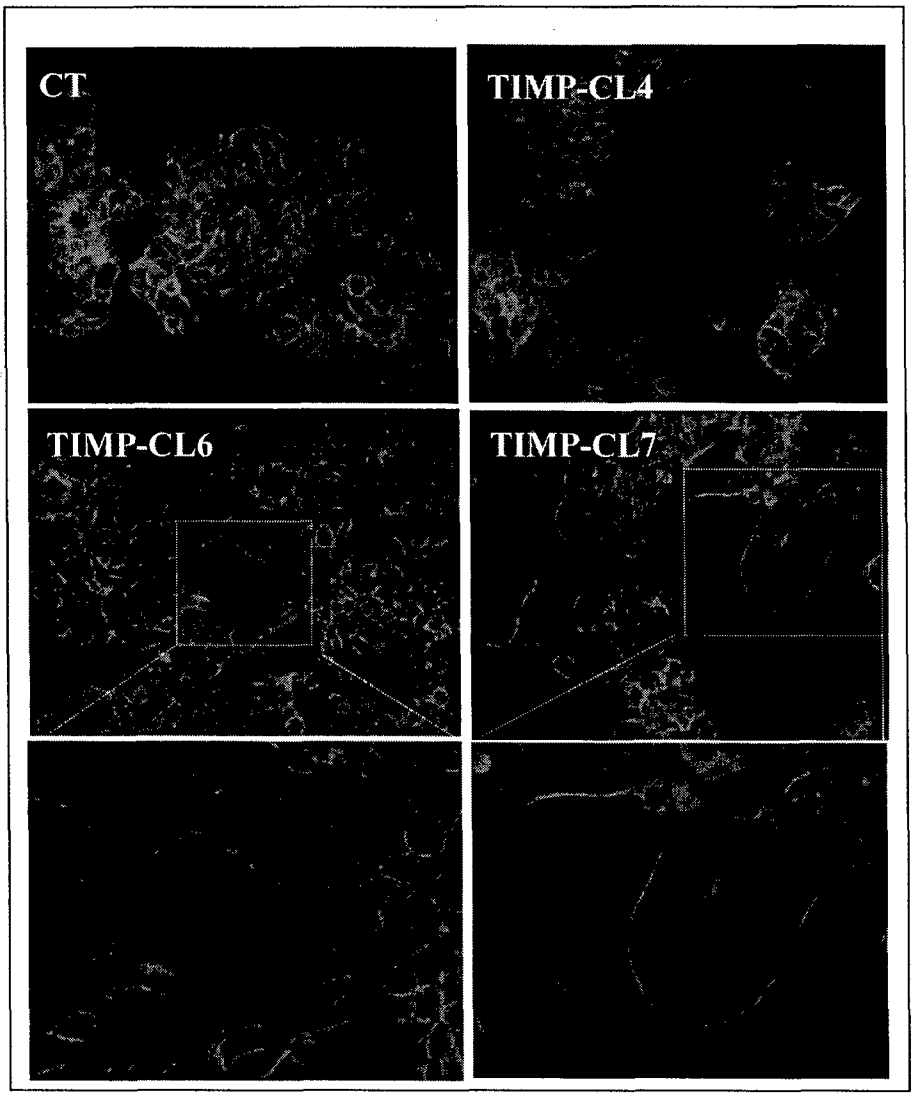

Figure 2 Control MCF-7 and three clones of TIMP-1 overpressors were stained to visualize nuclei (blue) and F-actin (green).